

Phased A-tracts bind to the α subunit of RNA polymerase with increased affinity at low temperature

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Abstract Previously we showed that the expression of a *Clostridium perfringens* phospholipase C gene (*plc*) is activated by promoter upstream phased A-tracts in a low temperature-dependent manner. In this paper we characterize the interaction between the α subunit of *C. perfringens* RNA polymerase and the phased A-tracts. Hydroxyl radical footprinting and fluorescence polarization assaying revealed that the α subunit binds to the minor grooves of the phased A-tracts through its C-terminal domain with increased affinity at low temperature. The result provides a molecular mechanism underlying the activation of the *plc* promoter by the phased A-tracts. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Curved DNA; α Subunit; RNA polymerase; Gene expression; Phospholipase C; *Clostridium perfringens*

1. Introduction

Many bacterial genes are controlled in response to changes in temperature. Changes in protein conformation, mRNA conformation [1], and membrane fluidity [2,3] are implicated in a thermosensing mechanism. A change in global DNA topology contributes to the thermoregulation of some virulence genes with the assistance of proteins that alter DNA superhelicity [1,4,5]. DNA local structures such as DNA curvature may also be involved in temperature-mediated gene regulation.

We found that three phased A-tracts forming intrinsically curved DNA are located between -66 and -40 relative to the transcription initiation site of a *Clostridium perfringens* phospholipase C gene (*plc*) [6]. We previously showed that the phased A-tracts stimulate the *plc* promoter activity in a low temperature-dependent manner [7,8]. Based on the fact that bending of the phased A-tracts increases with decreasing temperature [8], we suggested the low temperature-dependent stimulation is due to an increase in the bending angle, which facilitates the formation of a closed complex [7].

To elucidate the molecular mechanism underlying the low temperature-dependent stimulation by the phased A-tracts, we

addressed two questions: (i) which subunit of RNA polymerase (RNAP) do the phased A-tracts interact with?; and (ii) how does temperature affect their interaction? The finding that the α subunit of *Escherichia coli* RNAP (EcRNAP α) interacts with an AT-rich upstream (UP) element through its C-terminal domain (Ec α CTD) [9–11] may suggest a similar interaction between the three A-tracts and *C. perfringens* RNAP (CpRNAP). Thus, we analyzed, by means of a gel retardation assay and hydroxyl radical footprinting, the interaction of CpRNAP α or its α CTD (Cp α CTD) with the phased A-tracts. We also performed fluorescence polarization analysis with fluorescence-labeled DNA to examine the interaction between RNAP α and the phased A-tracts at various temperatures. The evidence presented here indicates that CpRNAP α binds to the minor grooves of the three A-tracts of the *plc* gene, and that the phased A-tracts exhibit increased binding affinity for CpRNAP α at a low temperature.

2. Materials and methods

2.1. DNA

3A_{plc} DNA, which contained the *plc* promoter and the phased A-tracts, was prepared as follows. A DNA fragment corresponding to -66 to +170 relative to the transcription initiate site was polymerase chain reaction (PCR)-amplified with pCM Δ T [8] and a set of primers, 5'-AAAAAATATTTTAAAAAATATTC-3' and 5'-ATCAATCTTCCATCCCAAGC-3'. The PCR product was cloned into the *EcoRV* site of the pT7Blue T-vector (Novagen), and the resultant plasmid, which was named pKMA308, was digested with *PvuII* and *AccII*. A 289-bp *PvuII*-*AccII* fragment was used as 3A_{plc} DNA. 0A_{plc} DNA was a derivative of 3A_{plc} lacking the phased A-tracts. Three 5'-fluorescein-labeled DNA fragments, 3A, 0A and UP DNAs, were 32-bp DNAs corresponding to the regions from -66 to -35 of 3A_{plc}, 0A_{plc} and the *E. coli* *rrnB* P1 promoter, respectively. These 32-mers, which contained a single primary amine at the 5'-end and were conjugated to fluorescein isothiocyanate, were obtained from Espec Oligoservice (Ibaraki, Japan). For the hydroxyl radical footprinting, pKMA308 was digested with *FokI*, and then a 326-bp *FokI* fragment containing 3A_{plc} was 5'-end-labeled with a Megalabe kit (Takara) and [γ -³²P]ATP. The fragment was also 3'-end-labeled with Klenow enzyme and [α -³²P]dTTP in the presence of 5 mM dCTP and 5 mM dGTP at 30°C for 15 min. The labeled fragments were digested with *AccII*, and then a 263-bp *FokI*-*AccII* fragment was used for footprinting analysis as 3A_{plc}.

2.2. Plasmid construction

The coding region of a *rpoA* gene, which encodes CpRNAP α (GenBank database accession number: AB034247), was PCR-amplified with *C. perfringens* NCTC8237 chromosome DNA as the template. The primers used were 5'-CATATGTTAGAAATAGAAAAGCCAG-3' and 5'-GGATCCTACTCGTCATTTAGTCTTAA-3' (*NdeI* and *BamHI* sites are underlined). The 956-bp PCR product was cloned into the *EcoRV* site of the pT7Blue T-vector, and the resultant plasmid was named pSK1. The regions corresponding to Cp α CTD

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Abbreviations: PCR, polymerase chain reaction; RNAP, RNA polymerase; RNAP α , α subunit of RNA polymerase; α CTD, C-terminal domain of α subunit; α NTD, N-terminal domain of α subunit

and the N-terminal domain of the α subunit (Cp α NTD) were PCR-amplified with pSK1 as the template and two sets of primers, 5'-GATTCATATGTTAGAAATAGA-3' and 5'-ATTAGGATCCTAAGTTAAAGTCATGAATAA-3'; and 5'-TGAGCATATGATTGAAAAGAAGAAGAT-3' and 5'-CCTCGGATCCTACTCGTCATTAGTCTTAA-3'. An EcRNAP α coding region was PCR-amplified with *E. coli* NovaBlue chromosome DNA and a set of primers, 5'-GGACCATATGCAGGGTTCTGTGACAGAG-3' and 5'-TGTGGATCCTTACTCGTCAGCGATGCTTGC-3'. All the PCR product was cloned into pET11a (Novagen) using *Nde*I and *Bam*HI, and then transformed into *E. coli* BL21(DE3)pLysS. All the fragments cloned into the plasmids were verified by nucleotide sequencing.

2.3. Purification of CpRNAP α , Cp α CTD and Cp α NTD, and EcRNAP α

All the *E. coli* transformants were grown at 37°C in LB medium containing 50 μ g/ml of ampicillin and 34 μ g/ml of chloramphenicol to the middle exponential growth phase, isopropyl β -D-thiogalactopyranoside was added to 1 mM, and cells were harvested after 3 h. To prepare CpRNAP α , cells were resuspended in buffer A (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol) containing 0.1 M KCl and 400 μ g/ml of phenylmethylsulfonyl fluoride (PMSF), and disrupted by French press treatment. The lysate was centrifuged at 30000 \times g for 30 min at 4°C. Cleared lysates were loaded onto a Q Sepharose Fast Flow column (bed volume 8 ml; Pharmacia). Proteins were eluted with a linear gradient of 0.1–1 M KCl in buffer A. Subsequent purification was performed as described by Fredrick et al. [12] except that buffer B (20 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 0.1 mM DTT, 10% (v/v) glycerol) containing 0.1 M KCl was used for separation on Sephacryl S-200 and MonoQ columns. The peak corresponding to CpRNAP α was collected, dialyzed against storage buffer (50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.25 mM DTT, 0.1 M KCl) containing 50% glycerol, and then stored at -80°C. 1 l of bacterial culture yielded 4.1 mg of purified CpRNAP α . The same procedure allowed the purification of Cp α NTD (1.6 mg/l of culture). Cp α CTD was obtained in the void volume of the Q Sepharose Fast Flow column, followed by dialysis against buffer B and chromatography on a MonoQ column. The peak eluted with 0.15 M KCl from a MonoQ column was used as purified Cp α CTD and the final yield was 2.6 mg/l of culture.

Cells expressing EcRNAP α were resuspended in buffer C (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 5% (v/v) glycerol) containing 0.1 M NaCl and 1 mM PMSF. The cleared lysate was loaded onto the Q Sepharose column and eluted with a linear gradient of 0.1–0.5 M NaCl in buffer C. The proteins eluted with 0.3 M NaCl were subjected to ammonium sulfate precipitation, gel filtration and ionic exchange chromatography as described above except that buffer D (10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 10 mM MgCl₂, 0.1 mM DTT, 5% (v/v) glycerol) was used. The final yield was 3.4 mg/l of culture.

2.4. Gel retardation assay and hydroxyl radical footprinting

The gel retardation assay was performed in the same manner as described previously [7]. Binding reactions were performed using various amounts of CpRNAP α or its domains and a constant DNA concentration (1 nM) in 30 μ l of binding buffer (12 mM HEPES (pH 8.0), 4 mM Tris-HCl (pH 8.0), 60 mM KCl, 1 mM EDTA, 1 mM DTT, 12% (v/v) glycerol, 0.3 mg/ml bovine serum albumin (BSA), 5 μ g/ml poly(dI-dC)) at 25°C for 15 min. The reaction mixture was loaded and run on a 4% polyacrylamide gel at 25°C, which was maintained during electrophoresis. The bands were visualized and analyzed with a BAS1500 Bio-Imaging Analyzer (Fuji Photo Film). For hydroxyl radical footprinting, the binding reaction was carried out under the same conditions as for the gel retardation assay except that glycerol was omitted from the buffer.

2.5. Fluorescence polarization DNA binding assay

The binding affinity of CpRNAP α for the phased A-tracts was measured by means of a fluorescence polarization DNA binding assay, which was developed based on the rationalization that the binding of proteins to their binding sites reduces the tumbling rate of the DNA, resulting in a large increase in number of the polarization units of fluorescently labeled oligonucleotides [13–16]. Polarization was measured with a Beacon 2000 fluorescence polarization system (Panvera, WI, USA). The annealed oligonucleotides (1 nM) were incu-

bated with various concentrations of purified proteins in 100 μ l of polarization binding buffer, which was the same as the binding buffer used for the gel retardation assay except that poly(dI-dC) and BSA were omitted. The mixture was incubated at the indicated temperature for 15 min and then transferred to a borosilicate glass tube (6 \times 50 mm). Polarization was read three times and fluorescence polarization values were averaged. The data for three experiments were plotted and fit to a hyperbola using the non-linear curve fitting functions of the MacCurveFit (Kelvin Raner Software, Australia).

3. Results

3.1. Characterization of the gene encoding CpRNAP α

Inspection of the deduced amino acid sequence of the *C. perfringens* *rpoA* gene revealed that CpRNAP α is a 35.1 kDa peptide of 315 amino acids, i.e. 14 amino acids shorter than the *E. coli* homologue (EcRNAP α). CpRNAP α exhibits 44% identity and 63% similarity to EcRNAP α . Based on the sequence similarity, we constructed two recombinant proteins, Cp α NTD (residues 1–228) and Cp α CTD (residues 237–315). CpRNAP α , Cp α NTD and Cp α CTD, and EcRNAP α were purified from recombinant *E. coli* cells to near homogeneity (data not shown). The molecular masses of the four constructs determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry coincided with those calculated from the deduced amino acid sequences (data not shown). The apparent molecular masses determined by gel filtration of CpRNAP α , Cp α NTD, and Cp α CTD were 84, 50, and 18 kDa, respectively, indicating that they each exist as a dimer in solution like EcRNAP α and its domains [17,18].

3.2. Binding of 3A_{plc} DNA to Cp α CTD

Using 3A_{plc}, and purified CpRNAP α , Cp α CTD and Cp α NTD, we performed a gel retardation assay (Fig. 1). CpRNAP α completely retarded 3A_{plc} at a concentration of 1.0 μ M. Cp α CTD also retarded 3A_{plc} at 15 μ M, indicating that Cp α CTD can bind to the phased A-tracts, although with lower affinity than in the case of CpRNAP α . Such a difference

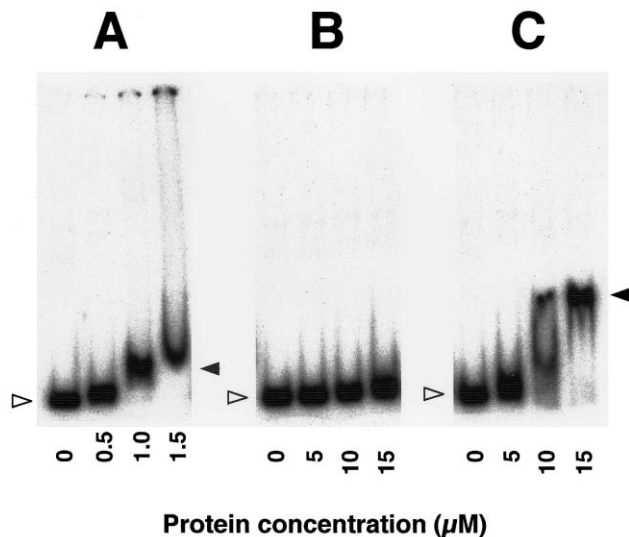


Fig. 1. Gel retardation assay for binding of CpRNAP α and Cp α CTD to 3A_{plc}. The ³²P-labeled 3A_{plc} (30 fmol) was mixed with 30 μ l of the reaction buffer containing various amounts of purified CpRNAP α (A), Cp α NTD (B), or Cp α CTD (C). The protein amount in the reaction mixture, which was expressed as the dimer concentration (in μ M), is denoted below each lane.

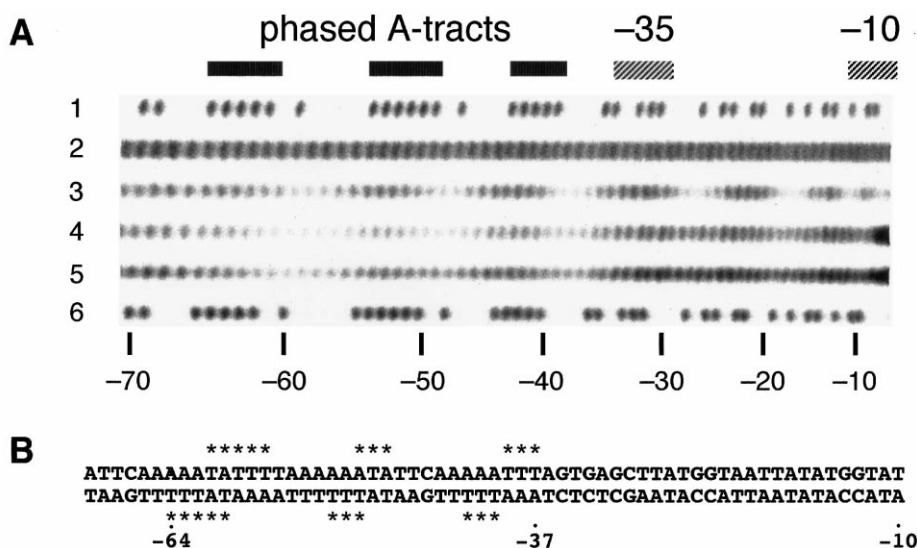


Fig. 2. Hydroxyl radical footprinting of CpRNAP α - and Cp α CTD-3A_{plc} complexes. A: Autoradiographs of hydroxyl radical footprinting of 3A_{plc} complexed with the purified proteins. Lanes: 1 and 6, A and G chemical sequencing ladder; 2, 3A_{plc} alone; 3, plus CpRNAP (16 nM); 4, plus CpRNAP α (1 μ M dimer); 5, plus Cp α CTD (15 μ M dimer). Only the footprint of the top strand is shown. The locations of the phased A-tracts, and the -35 and -10 elements are also shown by filled and shaded boxes, respectively. B: A map of protected nucleotides. Asterisks indicate the nucleotides protected by CpRNAP α from hydroxyl radical cleavage, which are identical with those protected by Cp α CTD and also with those in the promoter upstream region protected by CpRNAP.

has also been reported for the affinities of EcRNAP α and Ec α CTD to the UP element [17]. On the other hand, Cp α NTD did not cause any retardation of 3A_{plc} at 15 μ M (Fig. 1) or even at higher concentrations (data not shown).

In order to map the position of the contact region, CpRNAP α or Cp α CTD was complexed with 3A_{plc} DNA, and then analyzed by hydroxyl radical footprinting (Fig. 2). The two complexes showed the same footprint pattern: the three regions between -64 and -37 were protected. This protection pattern was also observed for the 5'-region of 3A_{plc} complexed with CpRNAP. The protected regions in the bottom strand were offset by two or three nucleotides compared with those in the top strand, indicating that Cp α CTD binds across the minor groove to the phased A-tracts [11].

3.3. Low temperature-dependent binding of CpRNAP α to 3A DNA

In order to examine a temperature effect on the binding of α CTD to the phased A-tracts or UP element, we determined the binding affinities of CpRNAP α to 3A DNA and 0A

DNA, and those of EcRNAP α to UP DNA at 25, 37 and 45°C (Table 1). The apparent dissociation constant (K_{app}) values of CpRNAP α -3A DNA binding are in the range of 0.16–1.1 $\times 10^{-6}$ M, while those of CpRNAP α -0A DNA binding were higher than 3 $\times 10^{-6}$ M, probably reflecting non-specific binding. The K_{app} value of CpRNAP α -3A DNA binding was lower than that of EcRNAP α -UP DNA binding at all temperatures. More importantly, the former decreased markedly with decreasing temperature. The latter also decreased slightly but significantly as the temperature decreased.

4. Discussion

The results presented here complement with the previous study involving the CpRNAP and the *plc* promoter, and substantiate further the contact region of the CpRNAP α -phased A-tracts complex. Moreover, we showed that their binding affinity increases as temperature decreases [11,19]. Recently, Yasuno et al. [20] found that the binding strength of Ec α CTD as to UP element derivatives is proportional to the extent of DNA curvature. They proposed that a minor groove width is narrowed by DNA curvature, which allows Arg265 of Ec α CTD to interact with both sides of the phosphate backbone. Considering a temperature effect on DNA curvature [8], their model provides a molecular mechanism which explains the increased affinity of CpRNAP α to the phased A-tracts at low temperature. It also well explains why the K_{app} value of EcRNAP α for the UP element which contains two phased A-tracts, A₄ and A₃, was significantly lower at low temperature than at high temperature (Table 1).

The phased A-tracts differ in the hydroxyl radical footprinting profile from the UP element: the protection is between -40 and -55 (-40 to -44 and -51 to -55) in the case of the UP element, while it is extended to further upstream in the case of the phased A-tracts. Similar extension of the protected region has also been reported for the *rrnB* P1 promoter, in

Table 1
Binding affinity of CpRNAP α to 3A or 0A DNA, and that of EcRNAP α to the *rrnB* P1 UP element

RNAP α	DNA	Temperature (°C)	K_{app} ($\times 10^{-6}$ M)
CpRNAP α	0A	25	> 3.0
		37	> 3.0
		45	> 3.0
	3A	25	0.157 \pm 0.032
		37	0.607 \pm 0.060
		45	1.052 \pm 0.350
EcRNAP α	UP	25	0.845 \pm 0.019
		37	1.350 \pm 0.019
		45	1.503 \pm 0.033

The apparent dissociation constant (K_{app}) was determined as described under Section 2, and was expressed as the dimer concentration. Data are the means \pm S.D. for the three experiments.

which the UP element was substituted with four phased A-tracts [10]. These observations raise a question as to how a RNAP α dimer binds to the three phased A-tracts. The protection of the three A-tracts may result from a mixed population of DNA molecules which differ in the regions occupied by CpRNAP α , or from short-lived CpRNAP α interactions with different regions of the same DNA molecule [10]. It may also be possible that a CpRNAP α dimer interacts not only with two proximal A-tracts in the same manner as but also with the most distal A-tract in a different manner compared to an EcRNAP α dimer with the UP element. The modulation of promoter activity by DNA curvature in response to a change in temperature has also been suggested for the enhancer of the adenovirus E1A promoter [21]. Thus, a thermosensitive change in DNA curvature may underlie other gene regulatory systems that involve the association of DNA binding proteins with DNA curvature.

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